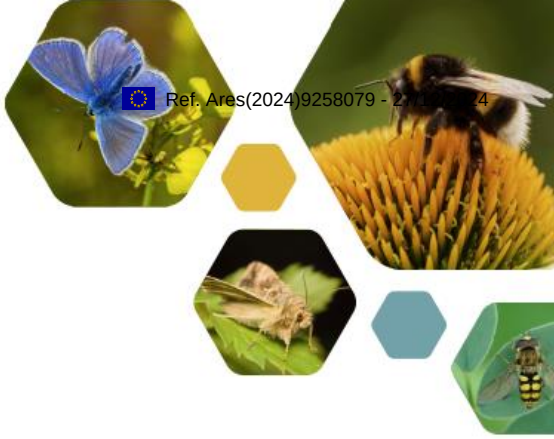

WildPosh

D1.1 A review of sampling protocols for nectar and pollen

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1 Summary

This deliverable focuses on reviewing the known methods for nectar and pollen sampling. The common sampling methods are described, and studies comparing the methods for different purposes are covered, but the focus is on comparing the methods from the perspective of studying pesticide residues in wildflowers.

In addition to reviewing the literature, a small field study was conducted in 2024 to compare certain methods, and the results of this study are presented and discussed. Finally, the relevant points for nectar and pollen sampling are discussed, bringing up best practices identified in the literature and by personal observations, as well as knowledge gaps present in the literature.

2 List of abbreviations

EU	European Union
HPLC	High-performance Liquid Chromatography
UHPLC	Ultra-High-Performance Liquid Chromatography
RP-LC-HRMS	Reversed Phase-Liquid Chromatography-High Resolution Mass Spectrometry
RPM	Revolutions Per Minute
RCF	Relative Centrifugal Force
USB	Universal Serial Bus

3 Introduction

Nectar and pollen are important nutritional resources for pollinators, and as such are often analysed for their nutritional properties in ecological research. Typical analyses include determining the sugar concentration or composition of nectar or the amount of lipids and protein in pollen [1], [2]. Other analyses may involve for example, plant secondary metabolites in nectar, or especially more recently the study of pesticide residues in nectar and pollen [3], [4].

To assess pesticide levels in nectar or pollen, these matrices must be sampled from the flowers and analysed. Ideally, there would be standardised methods for collecting the samples. Such methods would need to be easy to use in the field, yield samples that accurately reflect the pesticide residues accessible to pollinators and remain cost-effective.

However, developing a universally applicable sampling protocol poses challenges. Flower morphology, the amount, and characteristics of nectar and pollen, may differ between species and even among individual plants, making it difficult to develop a method that works consistently across all species.

When selecting a method, it is crucial to consider the requirements of the analysis, as different analytical goals may impose different constraints. For example, measuring sugar concentrations may require only a small amount of nectar, but the sampling method must avoid altering the concentration. Conversely, quantifying the relative amounts of different sugars in a sample is possible even if the sample is diluted.

The focus of this review is on applying the different sampling methods to analyse pesticide residues in nectar and pollen of wildflowers, considering the requirements the analysis methods pose to the samples. The review covers the most relevant literature on nectar and pollen sampling methods, introduces the methods available, and aims to identify best practices and potential knowledge gaps. Focus is given to studies comparing different methods, introducing new methods, or studies employing these methods specifically for pesticide residue analyses. Only sampling directly from flowers is considered, as bee-collected nectar or pollen is not in the scope of this review.

4 Nectar sampling

Descriptions of the most typical nectar sampling methods can be found in the book “Techniques for Pollination Biologists” [1]. Several articles have also compared nectar sampling methods for various analytical goals. Li et al. (2006) compared nectar collecting for the purpose of sugar concentration analysis, using multiple plants in the field, also examining the residual amount of nectar left in the flower after sampling by dissecting the flower [5]. Morrant et al. (2009) have tested several methods suitable for field sampling

and storing nectar from low-volume flowers, mostly for the purpose of analysing the sugar composition with High-performance Liquid Chromatography (HPLC) [6]. Power et al. (2018) also compared nectar collection methods suitable for low-volume flowers, with the emphasis on analysing the amino acid composition using Ultra-High-Performance Liquid Chromatography (UHPLC) [7]. Both comparisons came to a similar conclusion that results varied significantly based on the sampling method used. Perhaps the most relevant article in terms of pesticide residue analysis is by Pioltelli et al. (2024), who compared the most commonly used methods for analytical reliability, assessing the sugar and phytochemical profiles using Reversed Phase-Liquid Chromatography-High Resolution Mass Spectrometry (RP-LC-HRMS) [8]. A description of multiple methods for pesticide residue analysis is also available from Knäbe et al. (2015). No comparisons are made, implying that no differences in pesticide residues are assumed to result from using different methods [8].

As all methods are compromises in one way or another, and so the advantages and drawbacks alongside other relevant considerations are pointed out below where possible. Particular attention is placed on the versatility of the methods, whether the method is destructive or not, how easy it is to estimate the yield in the field, if the method retrieves an undiluted sample of nectar, and how efficient the method is. Versatile methods are preferred when sampling a range of different flowers, such as wildflowers. When analysing pesticide residues, it is important to retrieve undiluted samples of specific volume, making easy estimation of nectar yield in the field advantageous. Non-destructive methods are necessary when working at protected sites or when picking flowers needs to be avoided for other reasons. On the other hand, quantifying nectar volume per flower is not necessary for a pesticide residue study, even if it is very important when studying floral rewards.

4.1 Microcapillary tube method

The most common method to extract nectar from plants is through the use of glass microcapillary tubes [1]. The capillary tube is placed at the base of the flower nectary, and the nectar moves into the tube through capillary action (Fig. 1). The capillary tube is carefully moved around until no more nectar is gathered into the tube.

Nectar is relatively straightforward to extract with the microcapillary tubes, but some knowledge of floral morphology is required to place the capillary tube correctly. Even small flowers can be sampled if using small tubes, but handling small flowers requires a steady hand. Tubes in the range of 1µl to 20µl are said to work well for most flowers [1], although some flowers may require smaller ones, such as 0.25µl [9]. Extracting nectar with microcapillary tubes is a versatile method, allowing sampling from many types of flowers, but may not be suitable for the very smallest of flowers or for high-viscosity nectars [6]. Flowers may be sampled without picking them and nectar yield is easy to determine by measuring the length of the liquid column in proportion to the entire tube of known volume.

It is possible to bias the sample with tissue fluids if the tube is handled too aggressively [5], but otherwise the microcapillary tube method retrieves an undiluted sample of nectar and the nectar extracted this way is sometimes regarded as the best representative sample of raw nectar [7].

Extracting nectar from the microcapillary tubes may be done by attaching a rubber bulb to blow the contents out of the capillary tube. Another method is to place the capillary tubes into a centrifuge tube and carefully centrifuging them to extract the nectar.

Figure 1: A 2µl microcapillary tube placed in the nectary of a knapweed flower. Typically, the tube would be moved around slightly with the hand that is not used to hold the flower.

4.2 Micropipette or syringe method

Using a micropipette or a syringe is similar to using a microcapillary tube, except nectar can be aspired using the pipette or syringe mechanism, which may be helpful with some floral morphologies [5]. They are more cumbersome to handle and may therefore not be suitable for small and delicate flowers, and may not work effectively with low-volume flowers [6], but can be very efficient with flowers with larger nectar volumes.

Other than differences in versatility, similar positive and negative points can be mentioned about the micropipette or syringe method as for the microcapillary tubes; they are non-destructive, yield estimation is relatively easy, and the sample is undiluted. Pooling the sample into a centrifuge tube for storage is straightforward, and yield may be determined easily using the pipette or syringe or approximated visually in the tube. The sample is as good a representative of raw nectar as a sample collected with microcapillary tubes, unless it has been contaminated with tissue fluids.

4.3 Filter paper method

Another way of collecting nectar from the flower is by touching the nectary with a wick made from filter paper. The size of the wick can be adapted to allow many flower sizes but becomes difficult with very small or very large flowers [5]. With paper wicks the nectar can be sampled without picking the flower, making it non-destructive like the microcapillary tubes or micropipettes and syringes. With paper wicks it is difficult to damage the flower and contaminate the sample with tissue fluids, but it may be easily contaminated with pollen [5], [7]. The paper wick may catch very small drops of nectar more easily than a microcapillary tube [5].

Yield estimation in the field with the filter paper method is more complicated than with using the previous methods and requires preparations in the lab to be done beforehand. One method is to use standard-sized paper wicks and testing the column length a certain volume of nectar creates on the wick. The nectar yield can then be visually estimated by the column length in the field [5]. Using dried and pre-weighed wicks also allows measurement of the dry nectar constituents by weighing the wick again after sampling and drying [6].

The filter paper can be stored many ways depending on the analysis [10], but for many applications it is likely necessary to elute the nectar into a solvent, further complicating the calculations of nectar volume.

4.4 Centrifugation method

Nectar can be extracted from flowers by placing them face down over a filter or otherwise securing them at the top of a centrifuge tube, allowing the nectar to flow down when spinning the tube. Centrifugation can be used even when the flowers or nectar volumes are very small. It may also work when the flower morphology restricts the use of other methods. It is a destructive method, and therefore cannot be used when picking the flower is not allowed, or when multiple successive measurements from the same flower are required. Pollen or tissue fluid coming out of cut flowers may also contaminate the sample. Another drawback is the equipment required. Centrifuges small enough to be taken to field are likely small tabletop centrifuges, fitting typical 1.5ml and 2ml centrifuge tubes, restricting the size of flowers that can be sampled (Fig. 2). If whole plants can be collected and quickly transported to the lab, a larger lab centrifuge may be used. Nectar yield can be roughly visually estimated from the centrifuge tube, or more accurately by using capillary tubes. If using pre-weighed centrifuge tubes, the weight change can be used as a proxy for nectar volume.

As many of the previously mentioned sampling methods become more difficult to use as the flowers become smaller, a small field centrifuge may complement those methods well, as it is especially suitable for small flowers. Extracting nectar with a centrifuge is very fast compared to capillary tubes (see section 4.7). The sample obtained by centrifugation is not inherently diluted, but depending on the centrifugation settings and filters the sample

may become contaminated by tissue fluids, pollen, or other plant parts. An important consideration then is the appropriate centrifugation settings. Knäbe et al. (2015) uses a short centrifugation time of only 2-3 seconds and a typical small tabletop centrifuge [8], while Li et al. (2006) suggests using longer centrifugation time of 10 minutes at 4000 rpm (centrifuge type or rcf not given) [5].

To keep the flower in place, Li et al. (2006) uses a pin or a finger part of a disposable glove with small holes cut at the tip, which is then placed into a 1.5mL centrifuge tube [5]. Knäbe et al. (2015) uses an additional filter tube with 100 µl nylon mesh. Glass wool has also been used as a filter [11]. The methods by Knäbe et al. (2015) are likely similar to what are being used for pesticide testing by agrochemical companies [8].

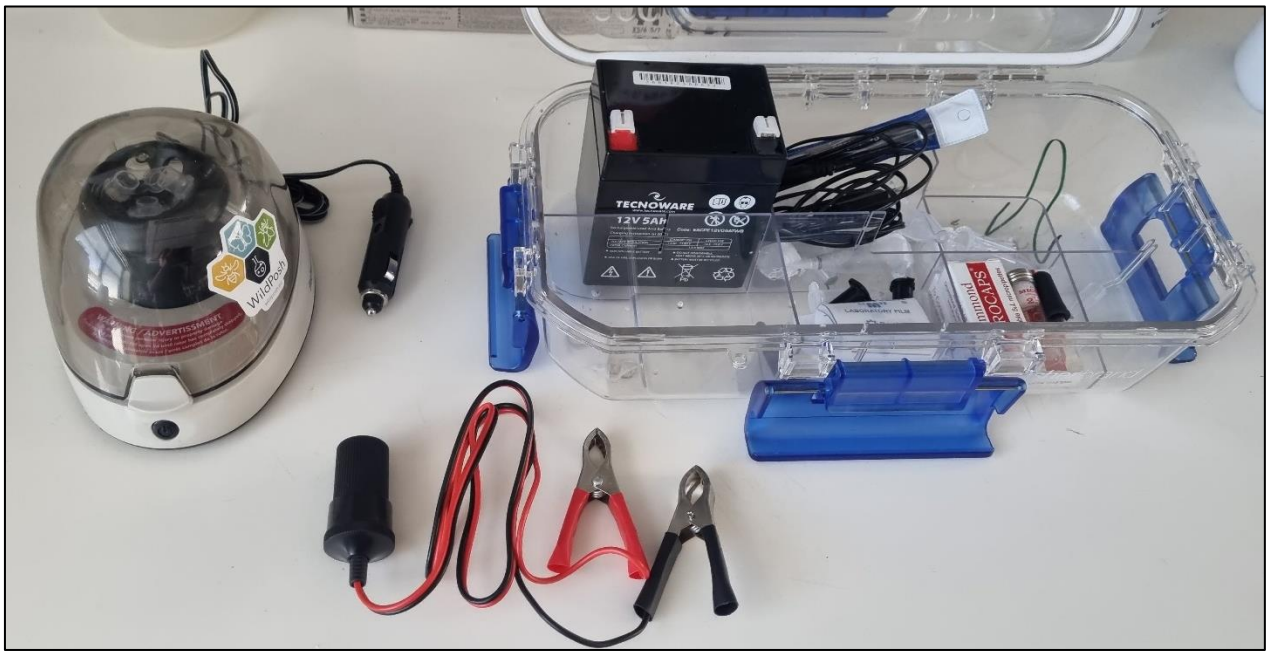


Figure 2: A field centrifuge setup. A small tabletop centrifuge is used with a car-adapter, a small battery, and an additional cable with a car plug. The centrifuge, battery, and the cables fit into the carrying case, which also has space for supplies like centrifuge tubes.

4.5 Wash method

The washing method consist of submerging the flower into a tube with distilled water, for example into a 20ml vial with 2ml of water [6], [7]. To extract nectar and other flower contents into the water the vial may be incubated or agitated. The method is described as quick and practical, and works well with small flowers, low nectar volumes, or when complex floral morphology makes other methods unsuitable [6]. Additionally, washing extracts sugars from a flower more effectively than other methods, making it a potential option for studying nectar sugars [6].

Morrant et al. (2009) compared different agitation durations and determined that an agitation of 1 min is the most suitable [6]. Additional washes may extract more sugars from the flower but is cumbersome in the field [6].

Extracting nectar by washing easily introduces pollen and other contaminants (including residues present on the surface of petals) into the sample and is not suitable for amino acid analysis [7]. The volume of nectar gained is also impossible to determine, and the sample is diluted with the amount of water used. If using the wash method, the yield would need to be estimated by assessing an average amount of nectar per flower using for example microcapillary tubes. As flowers need to be picked, the wash method is also destructive.

4.6 Other methods

Many of the nectar sampling methods mentioned previously can be coupled with rinsing the flower with water. In the rinsing method, a small volume of water is added to the flower nectary. The addition of water will help extract very low nectar volumes or nectar with high viscosity. The flower can be inverted over a collection vial, and then the flower nectary can be rinsed four successive times with 0.5ml of distilled water using a micropipette. The method may introduce pollen contamination and may not be suitable for amino acid assays [7]. Power et al. (2018) also compare a micro-rinse method, in which only 2µl of water was added to the nectaries and after 1min collected by a microcapillary tube. This method was deemed more suitable for amino acid analysis, as pollen contamination was easier to prevent [7]. The volume of nectar obtained is complicated to infer and requires knowledge of the average nectar volume per flower [7]. All rinsing methods necessarily dilute the nectar, making them less suitable for pesticide residue analysis.

For certain specific applications there are also methods not described above. Measuring sugar concentration with a refractometer is often done with microcapillary sampling [1], but an in vivo method has also been developed, using a thin fiber-optic probe, pointed directly to the plant nectary [12]. While clearly not suitable for other applications, it is a very interesting non-destructive tool for measuring sugar concentrations.

4.7 Comparison of efficiency

A small field study was conducted in 2024 to compare the efficiency of microcapillary tubes and the centrifuge method in gathering a nectar sample of 20µl, which represents an ideal sample amount to analyse wide variety of chemicals. The study was conducted at the Crop Research Unit fields at the University of Reading. Samples of red clover, *Trifolium pratense*, were taken with 2µl microcapillary tubes and the centrifuge setup shown in Fig. 2, using 30s spin time and 75µm metal inline filter [8]. The samples were stored in a cold box on ice packs in the field and at -20 °C in the lab.

4.7.1 Results

The centrifuge method was significantly faster in gathering a sample than the microcapillary tubes (Fig. 3a). The mean time to gather a sample using microcapillary tubes was 115.3 minutes, while with a centrifuge it was 25.7 minutes (Welch's t-test, $t = 5.27$, $df = 7.69$, $p < 0.001$). The required number of flowers was not significantly higher using microcapillary tubes (Fig. 3b), the mean amount being 34.1, while it was 9.7 using the centrifuge (Welch's t-test, $t = 1.94$, $df = 7.29$, $p = 0.09$).

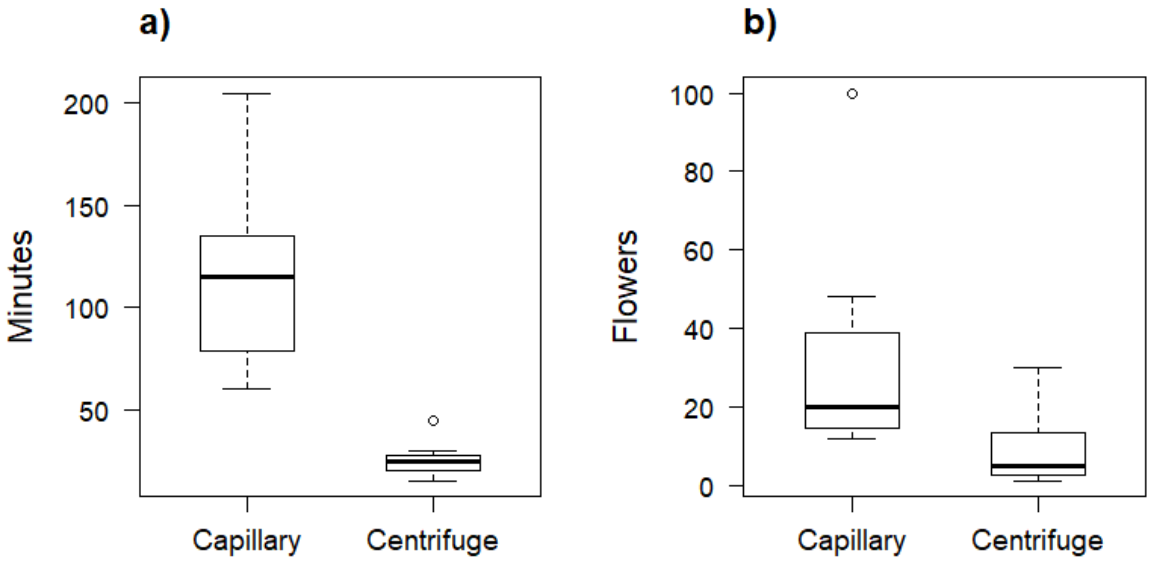


Figure 3: Differences in sampling efficiency of the microcapillary tube ("Capillary" n=8) and centrifuge ("Centrifuge" n=7) methods. A) Time required for one sample in minutes, B) Flowers required for one sample.

The samples were analysed for pesticide residues, but no residues were found in any of the samples. The fields in question were not sprayed with pesticides in 2023 or 2024, except with glyphosate, which was not among the analysed pesticides.

4.8 Nectar sampling method discussion

Certain methods, like the wash and rinse methods, require additional water to be inserted to the sample. The sample yield can only be inferred by studying the average nectar production in other flowers of the same species and age using other methods, such as microcapillary tubes [7]. The rinse and especially the wash methods seem therefore less suitable for pesticide residue studies. The paper wick method has the same disadvantage to a degree, as the sample needs to be eluted out of the filter paper and the volume of nectar collected can only be estimated through proxies like weight change.

Comparative studies have shown differences in amino acid, macronutrient, and phytochemical compositions as well as sugar extraction based on the nectar extraction methods. For studies on nectar sugar contents, Morrant et al. (2009) recommend washing or rinsing methods for low-volume flowers, as they extracted more of the sugars than other methods like the microcapillary tube [6]. The difference in sugar retrieval could be due to washing and rinsing dissolving dried sugar from the petal surfaces into the sample, which would not happen when using microcapillary tubes [7]. For amino acid studies on nectar, Power et al. (2018) prefer microcapillary tubes, because the other methods were more likely to contain pollen contamination, with the micro-rinse method being the best alternative for microcapillary tubes [7]. Centrifugation was not among the tested methods in these two articles, but was included in the comparison of methods by Pioltelli et al. (2024), who recommend microcapillary tubes and centrifugation as the best standard methods for nectar sampling, or when those are not suitable, the micro-rinse method [13].

Interestingly, they also report that the centrifuge extracted the least nectar compared to micro-rinsing and capillary tubes [14], which is contradictory to our results (Section 4.7). This discrepancy may partly be explained by different methods being more suitable for sampling different species, and by the fact that in our study thorough nectar extraction was not prioritised, likely favouring the centrifuge. Centrifugation settings like time and speed may also play a role in extraction efficiency, but these details were not reported [13]. How these details affect nectar sampling efficiency and the sample characteristics have not been properly studied.

In conclusion, microcapillary tubes and centrifugation appear to be the best suited methods for sampling nectar from a wide range of flowers for pesticide residue analysis. Other methods, like micro-rinsing, may also be considered depending on the analysis goals.

5 Pollen sampling

Pollen sampling methods have not been compared as much as nectar sampling methods. A description of common methods can be found in a book “Techniques for Pollination Biologists” [1]. A variety of pollen sampling methods is also described by articles from Vaudo et al. (2020) and an accompanying blog [2], [9]. Recently, Pioltelli et al. (2024) have compared methods in terms of their suitability for macronutrient profiles [14], as well as for the efficiency and reliability for more detailed phytochemical analysis using RP-LC-HRMS [13]. A description of a few methods specifically for pesticide residue analysis is available from Knäbe et al. (2015), although no comparisons are made between the methods [8].

As with nectar sampling methods, the advantages, drawbacks, and other relevant observations for each method are pointed out where possible below. The versatility, destructiveness, yield estimation in the field, sample purity, and efficiency of the methods are considered. Although efficiency is an important factor, sampling pollen for a pesticide residue study does not require collecting all the pollen from a flower. A representative sample sufficient for chemical analysis can be obtained using various methods, some of which are not designed to collect all the pollen from a flower.

5.1 Dried flower or anther sifting method

In many pesticide residue studies pollen is sampled by collecting flowers in the field, transporting to the lab and drying them in an incubator for 24 hours at 37°C [15], [16]. Drying facilitates pollen release from the anthers. It can then be released by brushing or rubbing and sifted through sieves with decreasing pore sizes to separate the pollen from other plant material (Fig. 4).

The method has been used to sample crop species like oilseed rape as well as wildflowers and ornamental plants [15], [15], [17], and is therefore quite versatile, although it is obviously also destructive. For species with very low pollen production, such as *Trifolium pratense*, separating the pollen from other dry plant material by drying and sifting is very challenging (pers. obs.).

A benefit of this method for crop species is that flowers can be collected before dehiscence, ensuring pollen availability as no pollinators have had access to the pollen, although collecting flowers before dehiscence may also exclude some pesticide residues resulting from spray drift. Wildflowers are likely to vary more in the flowering times, making collecting enough flowers before dehiscence more difficult.

As dry and brittle plant material is sifted along with the pollen, it is more likely to end up in the sample than with other methods. This can be prevented by using a sieve size closely matching the target pollen size or sieving only anthers rather than whole flowers. Collecting anthers for sieving would limit the destructiveness of the method, but also increase the time required in the field.

Another benefit of the method comes from the fact that flowers only need to be collected in the field, can be stored frozen, and the rest of the processing takes place in the lab at the researchers' own convenience. This also brings up an issue, as pollen yield is not known until after processing. The number of flowers required may be calculated if pollen production is known, but for wildflowers it may be difficult as the amount of pollen may vary by orders of magnitude between species [18].

Figure 4: Dried *Trifolium pratense* flowers on a sieve in a laboratory.

5.2 Anther ultrasonication method

Rather than drying the flowers or anthers, pollen can be separated from anthers in an ultrasonic bath [18]. Dehisced anthers are placed into a 1.5ml Eppendorf tube containing ethanol. The tube is vortexed, ultrasonicated in an ultrasonic cleaner, then vortexed again, and the ethanol containing pollen is collected [18]. These wash steps are repeated until no pollen is left on the anthers. The ethanol can be removed by pelleting the pollen with a centrifuge, removing the supernatant and then air drying.

The versatility, destructiveness and efficiency of this method can be assumed to be similar to the sifting method, and in addition the sample may be affected by the ethanol washes. This method has been used to measure pollen production and may not be suitable for chemical analyses of the pollen.

5.3 Vacuum collection method

Extracting pollen directly out of the flowers using a vacuum device has been done by attaching a filtered pipette tip to the suction hose of a vacuum-pump [8], or with a device based on a portable vacuum cleaner [14]. The vacuum pump is mentioned to be effective mostly for certain plants with sticky pollen, like in the family *Cucurbitaceae* [8]. The portable vacuum cleaner based device, called electronic pollen sampler (E-PoSa, Fig. 5) described by Pioltelli et al. (2023), has been shown to be versatile, as it has been tested with numerous flower species with varying morphologies [14]. As pollen is sampled directly from the flowers, they do not need to be collected, making vacuum sampling a non-destructive method.

With the electronic pollen sampler, the pollen is harvested directly into a 5ml Eppendorf tube, allowing a crude visual estimation of yield. Using pre-weighed collection tubes, the yield can be more accurately measured in the field with a precision scale. Although a specialised device needs to be constructed, the process is simple, and the parts are affordable. The device is straightforward and easy to use, making it an efficient pollen sampler. It has been described as low time-consuming and high-yielding, and capable of sampling pollen from meadow species that produce low amount of pollen [13]. However, our own experiences suggest that using it to collect the large amounts of pollen is still unfeasible (see section 5.8).

Figure 5: The electronic pollen sampler (“E-PoSa”), based on a USB powered portable vacuum cleaner (left) and the device in action (right).

5.4 Flower vibration methods

Vibrating the plants may be necessary to sample pollen from certain plants, such as the family *Solanaceae* [8]. The vibration imitates a visiting pollinator and helps release pollen.

This method may be necessary for plants that have poricidal anthers, which only release pollen when vibrated at a specific frequency. A vibrating tool may be constructed from an electric toothbrush [8], a tuning fork, or by a small loudspeaker attached to a device providing variable frequency output [1]. A container is placed under the flower to collect pollen falling from the flower [8].

Although some vibration may be necessary for certain flowers to release the pollen, a sonic dismembrator can release pollen from a wider variety of plants [19]. The sonic dismembrator is among the more versatile methods, it is non-destructive, the yield can be directly assessed with a scale, and it retrieves pollen samples free of other plant material or debris [19]. Despite its benefits it does not appear to be a widely used method, perhaps because it requires relatively expensive laboratory equipment costing several thousand pounds, that is also not intended for field use and requiring additional kit if brought to the field. The method can also be used destructively, if flowers are brought to the lab for processing. The efficiency of this method has not been compared, but it has been used to collect samples similar in size to other methods [16], [19].

5.5 Other fresh pollen collection methods

Collecting pollen directly out of the flowers may be accomplished in various ways that are more difficult to categorise under one method, as they have been used on a case-by-case basis depending on the sampled species. As mentioned above, certain plants may require vibration to release the pollen, and other methods may be applicable to only certain types of flowers. As they are group-specific, the versatility of these methods is limited.

5.5.1 Brushing

When pollen is directly accessible (e.g. sunflowers), it can be brushed gently into a container using a small brush with synthetic hair [2], [20]. The pollen may be further purified with sieves.

5.5.2 Shaking the flowers

Fresh pollen can be manually removed from wind pollinated plants such as maize by simply shaking the tassels over a container [20]. The pollen may again be further purified with sieves.

5.5.3 Tripping a pollen release mechanism

For keel shaped flowers that have a mechanism that releases the pollen, a collection vial may be placed around the keel petals in such a way that when the mechanism is tripped, the pollen is released into the collection tube [2], [21].

5.6 Collecting anthers

It may be helpful to remove the anthers before using any of the mentioned methods to extract the pollen from them, which can be done with fine tip tweezers [9]. The anthers can be collected to be dried and sifted in the laboratory (section 5.1) or separated “freshly” in the field with brushing or other methods. This approach may be useful for example with *Fabaceae* flowers that do not allow direct access to the pollen.

A method to help separate pollen from larger amounts of anthers in the laboratory using static electricity is described by Francis [9] (see also [2]). Dried or fresh dehisced anthers removed from flowers are placed in a plastic cup and vibrated using a vortex, which not only separates the pollen from the anthers but also makes the pollen stick to the sides of the plastic cup. The method has been used with varied floral groups [2] and is presumably versatile. The efficiency of this method is not known, but presumably collecting anthers is more time-consuming than collecting whole flowers, if compared to the flower drying and sifting method. The benefit is that collecting anthers is less destructive than collecting flowers.

5.7 Using anthers as a proxy

Pollen is a difficult matrix to sample in large quantities, and sometimes whole anthers are used together with pollen to achieve enough sample material [17], [22]. It is faster to collect whole anthers than just pollen and is likely applicable when pollen can also be sampled.

As the closest plant tissue, anthers are a sensible first choice as a substitute, and are likely to be the closest match for pollen in terms of pesticide residues [23]. Nevertheless, the pesticide residue dynamics between anthers and pollen differ, and the measured amount of active ingredient varies depending not only on the plant tissue but also on the pesticide used [23]. Residues may further be altered by the application methods of different pesticides [24], and it is then possible that using anthers as a proxy for pollen may over or underestimate the actual pesticide exposure for pollinators [23]. Anthers also differ from pollen in their nutritional and phytochemical composition [13]. It is therefore advisable to avoid using anthers as a proxy for pollen whenever possible.

5.8 Comparison of efficiency

During 2024 a small field study was conducted to compare the sampling efficiency of sifting dry flowers and the electronic pollen sampler. The target amount of pollen was 600mg, which represents an ideal sample amount to analyse a wide variety of chemicals, with losses and possible replication taken into account. The study was

conducted at the Crop Research Unit fields at the University of Reading. Flowers of corn marigold, *Glebionis segetum*, were collected into resealable plastic bags to be dried and processed in the laboratory. The electronic pollen sampler was constructed as instructed by Pioltelli et al. [14]. Two samples of approximately 250 flowers and one sample of approximately 1000 flowers were collected using both methods. The samples were stored in a cold box on ice packs in the field and in -20 °C in the lab. The amount of pollen was measured with a precision scale in the lab directly after processing, meaning that for the drying and sifting method the pollen had been dried, while the pollen collected by the electronic pollen sampler was not dried, biasing the results towards favouring the electronic pollen sampler.

5.8.1 Results

Only one sample of 600mg was obtained with the drying and sifting method (from approximately 1000 flowers), while no sample large enough was obtained with the electronic pollen sampler. To compare the methods, it was estimated how long it would take to gather a sample of 600mg based on the yield obtained with each sampling bout. It is notable that 600mg of pollen is a rather large amount of pollen, and as such it is not surprising that sampling amounts this high may be unfeasible.

It would take less time to gather one sample with the drying and sifting method compared to the electronic pollen sampler (Fig. 6a). The mean time to gather a sample by drying and sifting would be 11.0 hours, while with the electronic pollen sampler it would be 25.9 hours (Welch's t-test, t = -2.68, df = 4.00, p = 0.06). The mean number of flowers required using the sifting method would be 1812, while with the electronic pollen sampler it would be 14719 (Fig. 6b, Welch's t-test, t = -2.89, df = 2.05, p = 0.1). The differences here were not significant, but the power of the test was compromised due to low sample size (n=3 per method).

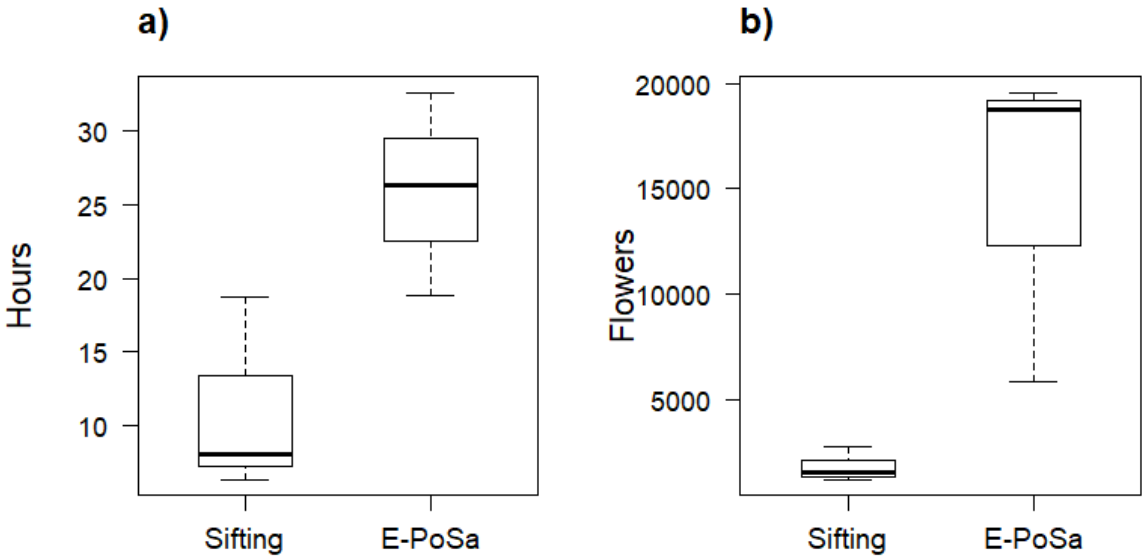


Figure 6: Differences in sampling efficiency of the drying and sifting method ("Sifting" n=3) and the electronic pollen sampler ("E-PoSa" n=3). A) Time required for one sample in hours, B) Flowers required for one sample.

A visual comparison of the samples under a microscope indicated that the electronic pollen sampler retrieved very pure pollen samples, while a small amount of plant material was evident in the sieved sample. The amount of plant material can be mitigated by using sieves that match the desired pollen size and by more careful sifting.

5.9 Pollen sampling method discussion

Collecting pollen samples appears to be more challenging than collecting nectar samples of sufficient quantity. This is shown by the fact that multiple studies also collect anther tissue rather than only pollen (e.g. [3], [17]).

In the comparison by Pioltelli et al. (2024) the electronic pollen sampler retrieved more pollen per flower than the drying and sifting method [14]. Interestingly again, in our field experiment we found contradictory results, as the drying method was the only method to gather enough sample material. It is unclear what causes the difference, but it may be due to availability of free pollen in different plant species or due to different sampling effort. Extracting all of the pollen by the vacuum method was not prioritised in our field experiment, possibly leaving pollen in the flowers. Sifting may also be done with varying effort. Aggressive sifting retrieves more of the pollen but likely also more unwanted plant parts, while more careful sifting retrieves a purer pollen sample, but some pollen may remain in the sieves.

Nevertheless, all sampling effort using the electronic pollen sampler happens in field conditions, while collecting flowers to be processed in the lab allows much more flexibility in time management. On the other hand, one needs to make sure to collect enough

flowers to be processed, whereas with the electronic pollen sampler the pollen yield can be measured in the field. When compared for analytical reliability, both methods result in comparable samples and could then be also recommended for pesticide residue studies, although the sampling efficiency remains unclear [13]. The vibration method using a sonic dismembrator seems intriguing, although the high cost of the equipment may be inhibiting. It has been successfully used to gather contaminant-free pollen from a variety of wildflowers [19] but has not appeared in comparisons in terms of efficiency or analytical reliability. Based on the literature available, these three methods all seem generally suitable for sampling pollen from wildflowers for pesticide residue analysis. However, some questions remain regarding sampling efficiency. To conduct a pesticide residue study with a wide range of chemicals and low limits of quantification, ideally at least 300mg of pollen is required per sample. This would be considerably difficult with any of the tested methods. A smaller sample of e.g. 50mg is more feasible but poses a compromise in terms of chemicals analysed and limits of quantification. We therefore recommend testing the methods beforehand in any particular study system.

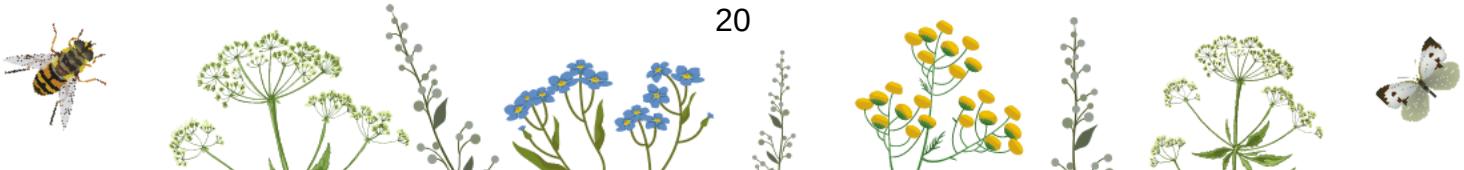
6 Knowledge gaps

In the context of pesticide studies the most notable gaps in knowledge surrounding nectar and pollen sampling methods are whether the selected method affect the measured pesticide residues in the samples. The studies on sugar, amino acid, or macronutrient or phytochemical profiles all indicate that sampling methods have demonstrable differences in the results [6], [7], [13].

When extracting nectar with the centrifuge there are details that might further change the properties of the extracted nectar. These details are the centrifugation force, centrifuge time, and notably what kind of filter is used in the centrifuge tube. The role of the filter is to allow nectar through, while other material stays on the other side. With low centrifugation forces and short centrifugation times, it might be assumed that mostly nectar is extracted. With increasing centrifugation forces and times, also pollen and tissue fluids may get extracted, also depending on the pore size of the filter. There are no studies regarding the effects of these centrifugation details, or the filters used, on any of the nectar characteristics.

Regarding pollen sampling, the drying and sifting method seems to retrieve similar samples as the vacuum method, but some questions remain around the sampling efficiency of these methods. Further studies on the sampling efficiencies and reliability for pesticide residue studies, also incorporating the sonic dismembrator method, would be beneficial.

Sample storage is another factor to consider when sampling plant matrices, while not the focus of this review. For nectar sugar analyses best results are obtained by refrigeration and doing the analysis within two weeks [6]. For other nectar components



like amino acids or pesticide residues a similar comparison is lacking. A study on pollen storage methods has also not been done. Keeping the samples on ice in the field and in -20°C in the lab is currently assumed to be a satisfactory method for storing samples for pesticide residue studies.

7 Conclusions

Several methods exist for both pollen and nectar sampling. The most common method for nectar sampling is by extracting the nectar using microcapillary tubes, which remains a recommendation. A complementary method for the microcapillary tubes is extracting the nectar using a centrifuge, as it allows sampling of the very smallest of flowers and low-nectar volumes, when sampling with microcapillary tubes would be difficult or time consuming. Both methods are likely to extract samples of good quality for pesticide residue studies.

Sampling pollen may pose more difficulties than sampling nectar, evidenced by the variety of methods developed to sample pollen from different types of plants and the fact that some plants produce tiny quantities of pollen. Sampling flowers to be dried and then sifting to separate the pollen is likely a suitable method for most situations, but the electronic pollen sampler and vibrating the flowers with a sonic dismembrator may be good or better alternatives, and likely provide good quality samples. However, the amounts of pollen required for pesticide residue studies may pose too great a challenge, regardless of sampling method.

Some knowledge gaps exist, most notably that none of the methods have been compared in terms of how reliably they can be used to sample nectar or pollen for pesticide residues analysis. It would be beneficial to compare the recommended methods in a controlled manner specifically for pesticide residue studies, also considering different centrifugation settings.

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